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Purification and identification of bovine liver γ-carboxylase

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ABSTRACT **The microsomal γ-carboxylase catalyzes modification of a limited set of glutamyl residues to γ-carboxyglutamyl residues in a vitamin K-dependent reaction that also utilizes O_2 and CO_2. We report the purification to apparent homogeneity of the bovine liver microsomal carboxylase. Affinity chromatography exploiting the association of the carboxylase with prothrombin precursor and carboxylase binding to the propeptide sequence were combined with ion-exchange chromatography and fractionation using immobilized lectins. A 3.5×10^5-fold purification was obtained, which is the highest purification, by a factor of 35, yet reported for this enzyme. A single 98-kDa protein is obtained from this isolation. Carboxylase activity is associated with this protein by two different criteria. Antibodies prepared against the carboxylase detected the 98-kDa protein when used in Western analysis. In addition, the single 98-kDa protein was shown to comigrate with activity when electrophoresed in a nondenaturing gel system. The availability of purified preparations of carboxylase will facilitate an increased understanding of the complex biochemical reaction carried out by this protein.**

The liver microsomal carboxylase catalyzes the posttranslational modification of selected glutamyl residues to γ-carboxylated glutamyl (Gla) residues in a limited set of proteins in a reaction requiring vitamin K hydroquinone, O_2, and CO_2 (1–3). These vitamin K-dependent proteins contain multiple glutamyl residues clustered at the N terminus in what is referred to as the Gla domain. Carboxylation of glutamyl residues in the Gla domain enables the Ca^{2+}-mediated interaction of these proteins with phospholipids and is required for their biological activity. Vitamin K-dependent carboxylase activity has been detected in almost all mammalian tissues assayed and has been observed in one invertebrate as well (4). Its apparent presence in a wide variety of cultured lines can also be inferred from the ability of these lines to secrete carboxylated vitamin K-dependent recombinant proteins (5–10).

A major limitation in understanding the mechanism of carboxylation has been the lack of a purified preparation of this enzyme. Attempts made using selective detergent extraction, ammonium sulfate fractionation, and conventional chromatography have been successful only in a limited purification (11–13). More recently, the propeptide sequence unique to all vitamin K-dependent proteins has been used in attempts to purify the carboxylase (14–16). This sequence is similar among vitamin K-dependent proteins that share very little other homology, leading to the proposal that the propeptide sequence is important for carboxylase recognition (17). The observations that mutations in protein C (18) or factor IX (19) impair carboxylation supported this concept. Carboxylase purifications have been attempted by using the propeptide as a ligand in affinity chromatography (15, 16). The enzyme has also been isolated by virtue of its known association with its vitamin K-dependent protein precursors (13, 20, 21). A peptide representing human factor X propeptide was used to elute carboxylase activity from α-prothrombin (α-PT)-Sepharose (14). However, only a 500-fold purification was obtained, and gel analysis did not reveal obvious carboxylase candidates. We have now purified the carboxylase to apparent homogeneity. Identification of the carboxylase has been established, using antibodies against the carboxylase as well as gel electrophoresis under nondenaturing conditions. The methods for identification are unrelated to those used in the purification and thus provide independent verification that the protein purified is the carboxylase.

MATERIALS AND METHODS

Purification of the Carboxylase. Microsomal protein (4.5 g) from warfarin-treated bovine liver (14) was resuspended in 70 ml of 20 mM Tris·HCl, pH 7.3/0.1 M NaCl and then centrifuged at 105,000 × *g* for 60 min at 4°C. Pellets (4.5 g) were resuspended in 70 ml of 20 mM Tris·HCl, pH 7.3/0.1 M NaCl and solubilized with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma). After 30 min at 4°C, the suspension was centrifuged as described above. The pellet (1.5 g) was resuspended in 70 ml of 20 mM Tris·HCl, pH 7.3/1 M NaCl/1% CHAPS. After 30 min at 4°C, the suspension was centrifuged as described above, and the supernatant was incubated with α-PT resin (35 ml) prepared as described (14). Resin and sample were rocked for 16 hr at 4°C and then poured into a column. Bound material was washed with 5 column vol of 20 mM Tris·HCl, pH 7.3/100 mM NaCl/0.5% CHAPS/0.5% phosphatidylcholine (III-E; Sigma)/5 mM dithiothreitol (buffer A). An additional column volume of buffer A containing 1 mM ATP and 5 mM $MgCl_2$ was added, column flow was stopped, and the column was incubated for 30 min. The resin was then washed with an additional 4 column vol of buffer A with ATP and $MgCl_2$. All column washes were performed at 20°C. Aliquots of detergent-solubilized material, flow-through, and α-PT resin were assayed for carboxylase activity (described below). Carboxylase was eluted from the resin with buffer A containing a propeptide (100 μM) comprising the −18 to −1 sequence of human factor X, using four batchwise incubations of the resin with propeptide. Each incubation was for 6–8 hr at 20°C, and ≈70% of the carboxylase was eluted from the column with the propeptide.

Propeptide eluant (200 ml) was adsorbed onto 0.5 ml of S-Sepharose (Pharmacia) at 4°C by rocking the two together for 16 hr. The resin was washed with 10 column vol of buffer B (50 mM Tris·HCl, pH 7.4/100 mM NaCl/0.25% phosphatidylcholine V-E/0.25% CHAPS) and the carboxylase was eluted by rocking the resin for 1 hr in buffer B (0.5 ml) adjusted to 0.2 M NaCl followed by incubation for a further 4 hr in buffer B (total of 1 ml) adjusted to 0.5 M NaCl.

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Abbreviations: Gla, γ-carboxyglutamic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; α-PT, α-prothrombin.

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S-Sepharose eluted material was adjusted to 5 mM $MnCl_2$ and 5 mM $CaCl_2$ and then adsorbed onto 200 μl of lentil lectin-Sepharose (Sigma), which was prewashed to remove any unbound lectin, for 16 hr at 4°C. Bound material was washed with a 100-fold excess of buffer B and carboxylase was then eluted over a 16-hr period in 1 ml of buffer B containing 0.5 M methyl α-mannoside (Sigma) and 10 mM EDTA. Approximately 50% of the carboxylase was bound to the resin. Using longer incubation times or more lentil lectin resin did not increase the percentage carboxylase bound to the resin. Of the material bound to lentil lectin-Sepharose, 50% was eluted with methyl α-mannoside. Attempts to reelute the lentil lectin eluant or to use other sugars (e.g., methyl α-glucoside) were unsuccessful in increasing the recovery of eluted activity.

Protein Characterization. Samples were analyzed on discontinuous SDS protein gels (8%; 38:1, acrylamide/bisacrylamide; Boehringer Mannheim). Protein was visualized with Coomassie dye (Sigma) using two cycles of staining on the propeptide eluant, S-Sepharose eluant, and lentil lectin eluant to increase the sensitivity of detection. Lentil lectin eluant was also silver stained (Daiichi kit) after pretreatment of the gel with periodic acid to enhance glycoprotein staining. Carboxylase samples were also electrophoresed on nondenaturing, continuous acrylamide gels. Samples were mixed with glycerol (to 10%) and bromophenol blue (to 0.025%) and electrophoresed through 8% (38:1, acrylamide/bisacrylamide) gels in 400 mM Tris·HCl, pH 8.8/0.25% phosphatidylcholine V-E/0.25% CHAPS, which had been polymerized with *N,N,N′,N′*-tetramethylethylenediamine and ammonium persulfate 1 day before and stored at 4°C. The running buffer contained 20 mM Tris·HCl, pH 8.8/1.4% glycine/0.1% phosphatidylcholine V-E/0.1% CHAPS. Aliquots of proteins ranging in size from 22 to 150 kDa were also run to monitor the gel resolution. In one experiment, radiolabeled lentil lectin eluant was also electrophoresed on the nondenaturing gel. After electrophoresis at 40 V for 16–20 hr at 4°C, the gel was cut into segments. One part was silver stained. Another part was fragmented into ≈20 pieces, each of which was incubated in the carboxylase assay (see below) for 1 hr. The lanes containing radiolabeled lentil lectin eluant were also fragmented. Protein was recovered from the peak of radioactivity by electroelution and then run on a denaturing gel as described above.

The high concentrations of propeptide that were present in several of the purification steps precluded using conventional methods for determination of protein concentration. To assay protein separated from the propeptide, aliquots of known amounts of carboxylase activity from each stage of the purification were electrophoresed on an SDS discontinuous gel and stained with Coomassie dye. Aliquots of bovine serum albumin were also run, and protein concentrations were quantitated on an LKB laser densitometer. The first two steps of the purification—i.e., the 0.5% and 1% detergent-solubilized microsomes that did not contain propeptide—were assayed by using the gel scanner as well as by using a BCA analysis kit (Pierce), and similar results were obtained.

Protein samples were radioiodinated using Iodo-Beads (Pierce). Carboxylase (1–10 ng) or affinity-purified rabbit anti-mouse IgG (Cappel) (50 μg) was incubated with 0.5 mCi of ^{125}I (1 Ci = 37 GBq) (Amersham) and one Iodo-Bead for 15 min at 4°C and then passed over a G-25 Sepharose column. Specific activities of 2–5 × 10^7 cpm/μg were obtained for each protein.

Carboxylase activity was determined by incubating protein aliquots in a 228-μl vol of 0.9 M ammonium sulfate/0.04% CHAPS/0.04% sodium cholate/0.04% phosphatidylcholine III-E/4 mM dithiothreitol/$NaH^{14}CO_3$ (20 μCi; 400 nmol; Amersham)/0.9 mM Boc-Glu-Glu-Leu-OMe (EEL, Bachem). After addition of vitamin K hydroquinone (22) (5 μg), the samples were rocked at 20°C, usually for 1 hr. Protein was then precipitated with 10% trichloroacetic acid (1 ml) and after centrifugation the supernatant was transferred to a scintillation vial, boiled to remove $^{14}CO_2$, and counted in 10 ml of BioSafe (Research Products International). To detect *in vitro* labeled vitamin K-dependent proteins, samples were incubated in the carboxylase reaction mixture and then gel electrophoresed. Gels were treated with Amplify (Amersham) and then dried and subjected to autoradiography.

Antibody Production and Protein Analysis. Propeptide-eluted carboxylase (8 × 10^6 cpm in 50 ml) was adsorbed onto 1 ml of S-Sepharose, washed with 10 vol of buffer B, mixed with an equal volume of complete Freund's adjuvant (23), and then injected interperitoneally into five BALB/c mice. The mice were given booster injections at 2-week intervals and after the third injection of antigen, mouse serum was tested along with control serum from a nonimmunized mouse. An activity immunocapture assay was used to detect α-carboxylase activity. Increasing amounts (0–20 μl) of sera were incubated with bovine microsomes (50 μl; 1.5 mg of protein) for 8 hr at 4°C, followed by addition of 50 μl of a 1:1 mixture of protein A-Sepharose (Sigma) in 50 mM Tris·HCl, pH 7.4/100 mM NaCl. Purified carboxylase (i.e., lentil lectin eluant) was also tested. Samples were rocked at 4°C for an additional 12 hr, and the resins were washed and then incubated in the carboxylase reaction mixture and processed as described above. Both nonimmune and immune serum samples were assayed in duplicate.

Lentil lectin-eluted carboxylase (10^5 cpm) was electrophoresed on an 8% SDS discontinuous gel and then transferred to nitrocellulose (Gelman Biotrace NT) at 60 V over 24 hr at 4°C in 1.4% glycine/25 mM Tris·HCl, pH 8.8/20% methanol/0.005% SDS. Western analysis was carried out in parallel with either nonimmune serum or α-carboxylase antiserum, using a 1:1000 dilution of serum and 2 × 10^6 cpm of ^{125}I-labeled rabbit anti-mouse IgG. Lentil lectin eluant and S-Sepharose eluant were also tested for the presence of prothrombin with a 1:1000 dilution of rabbit polyclonal anti-bovine prothrombin antibody.

RESULTS

Purification of the Carboxylase. Microsomes were processed through two detergent extractions before the first chromatographic step. Cycling the microsomes through a low (0.5%) and then a high (1.0%) concentration, CHAPS extraction produced only a 2- to 3-fold increase in specific activity, but it was significant in removing proteins in the 90- to 100-kDa range where the carboxylase was ultimately observed (Fig. 1). Detergent-solubilized microsomes were adsorbed to α-PT-Sepharose, washed, and eluted with a peptide corresponding to the human factor X propeptide sequence. This step, which comprises two affinity purifications—i.e., both antigen–antibody interaction and propeptide–carboxylase recognition—yielded a 1.4 × 10^3-fold increase in specific activity, with a 15% recovery. This low recovery largely reflected the limited amount of carboxylase–prothrombin complex; only 20% of the microsomal carboxylase was adsorbed to α-PT-Sepharose under conditions in which antibody was clearly in excess. No prothrombin was detected in the propeptide eluant, either by Western analysis or by *in vitro* carboxylation and gel analysis. The latter assay would also detect other vitamin K-dependent proteins, but none was observed.

In initial preparations of propeptide eluant analyzed on gels, the most prominent band observed had a molecular mass of 78 kDa. This protein, upon amino acid sequencing, was identified as BiP (also known as the 78-kDa glucose-regulated protein), a major endoplasmic reticulum resident protein (24). To remove this contaminant, α-PT-Sepharose

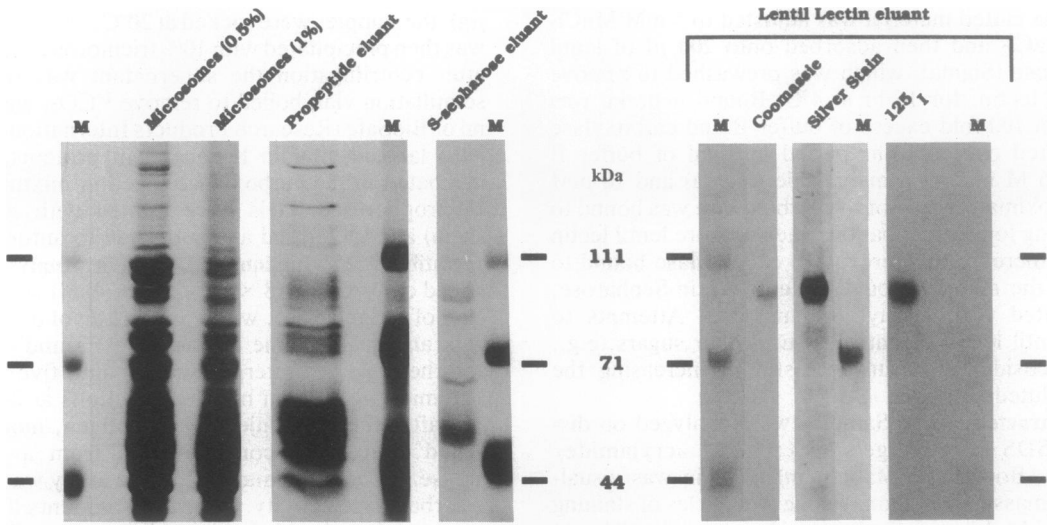

FIG. 1. Gel analysis of carboxylase at each purification step. Aliquots of protein at each stage of the purification were electrophoresed, along with prestained markers (lanes M; Bio-Rad). The units of activity (cpm/hr) were as follows: 0.5% microsomes, 2×10^4; 1% microsomes, 2×10^4; propeptide eluant, 6×10^4; S-Sepharose eluant, 2×10^5; lentil lectin eluant, 8×10^5. The gel sample containing lentil lectin eluant was Coomassie stained, photographed, and then silver stained. Most of the markers reverse stained during the silver staining.

was washed in an ATP/$MgCl_2$-containing buffer. As described for small peptides (25), ATP hydrolysis was associated with the release of BiP, and this procedure removed any detectable levels of BiP.

The carboxylase in the propeptide eluant, as well as in the crude and detergent-solubilized microsomes, was stable to multiple freeze–thaws and storage of these preparations at 4°C. Carboxylase purifications were routinely carried out without adding any protease inhibitors. Presumably, this stability of the carboxylase is due to protection by the lipid vesicle in which it is embedded. The organization of the carboxylase in a lipid vesicle may also explain an unusual feature observed during purification, which was the extended lengths of time required to achieve optimal recoveries. Carboxylase adsorption onto α-PT-Sepharose, for example, was performed over 12–16 hr and propeptide elution was carried out in several batches over 2 days.

The isolation of carboxylase using multiple batch elutions with propeptide generated a large volume (600 ml for the material summarized in Table 1) of very dilute carboxylase. An ≈200-fold concentration was achieved, however, by S-Sepharose chromatography. Carboxylase activity recovery was quantitative (Table 1) and a 12-fold increase in specific activity was achieved with this step.

A substantial enrichment of carboxylase was achieved by lectin chromatography. When carboxylase was fractionated on lentil lectin-Sepharose, activity was retained on the resin. Because the loading material (i.e., S-Sepharose eluant) contained no detectable vitamin K-dependent proteins, carboxylase binding to the resin did not appear to be indirect binding via a carboxylase–glycoprotein complex. The carboxylase, then, appears to be a glycoprotein containing the mannosyl residues common to all N-glycosylated proteins (26), which would be recognized by lentil lectin. Only ≈50% of the carboxylase was bound to the lentil lectin-Sepharose. Nonquantitative adsorption was not due to a subpopulation of unglycosylated carboxylase since lentil lectin-Sepharose flow-through could be adsorbed to fresh lentil lectin (data not shown). Moreover, 100% of carboxylase activity could be adsorbed to concanavalin A-Sepharose, which has a much higher affinity than lentil lectin for mannosyl residues (27). Unfortunately, carboxylase activity could not be recovered at all from concanavalin A-Sepharose by using methyl α-mannoside and/or methyl α-glucoside (data not shown).

When lentil lectin eluant was analyzed by gel electrophoresis, a single 98-kDa band was observed after Coomassie staining (Fig. 1), Ponceau S staining, or amido black staining (data not shown), or by radioiodinating the carboxylase and analyzing the products by autoradiography. When the lentil lectin eluant was analyzed by silver stain, the 98-kDa band was predominant, although two smaller bands of extremely low abundance (<2% of total protein) were also observed. The specific activity of the lentil lectin eluant was 3×10^7 cpm per hr per μg of protein, representing a 3.5×10^5-fold purification over the detergent-solubilized microsomes (Table 1).

Identification of the Carboxylase. *Antibody production and analysis.* Anti-carboxylase antibodies were prepared in mice and tested in an immunocapture assay (Fig. 2). About 2–3% of total carboxylase activity was captured by 20 μl of serum, and this amount increased ≈2-fold with subsequent antigen boosts. The small amount of reactivity likely reflects the low α-carboxylase titer obtained because of the small amounts of protein injected into the mice. When antiserum was used in

Table 1. Purification of the bovine liver γ-carboxylase

Sample	Protein, μg	Activity, cpm/hr	Specific activity, cpm·hr^{-1}·μg^{-1}	Purification, -fold	Overall recovery, %
Detergent-extracted microsomes	4.5×10^6	3.9×10^8	87	—	—
Propeptide eluant	5.0×10^2	6.0×10^7	1.2×10^5	1.4×10^3	15
S-Sepharose eluant	43	6.0×10^7	1.4×10^6	1.6×10^4	15
Lentil lectin eluant	0.6	1.8×10^7	3.0×10^7	3.5×10^5	5

The carboxylase purification represented here has been performed approximately a dozen times and the values given in this table are the sums of three different preparations. The activity recovered in propeptide eluant differs from that reported (14). This is due to the different assays and to the use here of more optimal propeptide concentrations [100 μM versus 1 mM (14)], of resolubilized microsomes, and of more extensive washing conditions for the α-PT-Sepharose.

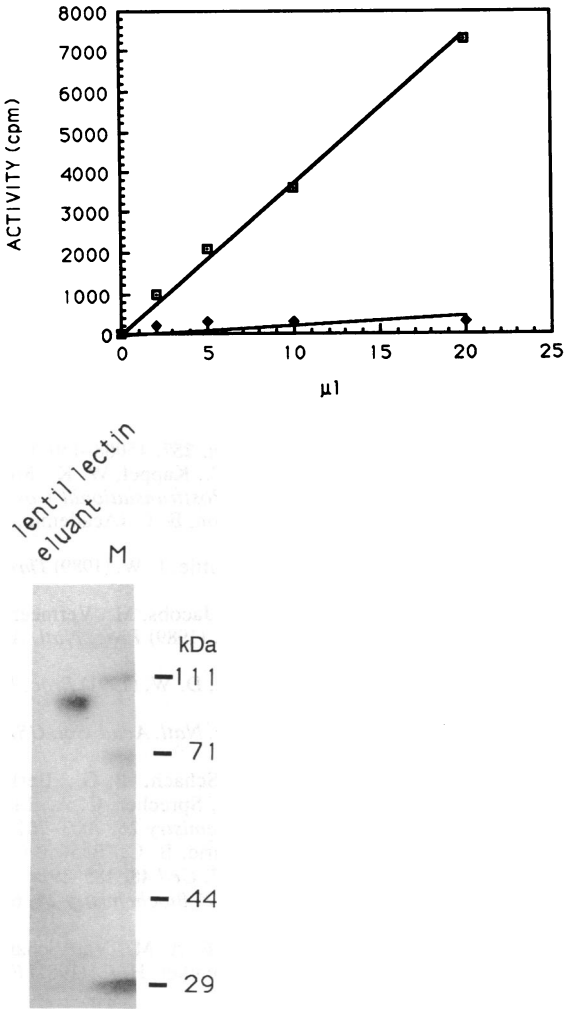

FIG. 2. Western analysis of purified carboxylase. (*Upper*) Anti-carboxylase reactivity was tested by using an immunocapture assay, comparing antiserum from a mouse injected with carboxylase (□) with nonimmune serum (◆). (*Lower*) Anti-carboxylase antiserum was used to probe the lentil lectin eluant in a Western analysis. The radiolabeled 44-kDa marker is not visible in the exposure.

a Western analysis of lentil lectin eluant, a 98-kDa band was detected (Fig. 2). When S-Sepharose eluant was analyzed by Western blotting, this 98-kDa band and several other bands were observed (data not shown). By contrast, nonimmune serum did not detect any protein in either the S-Sepharose or the lentil lectin eluant. These two eluants were also tested for the presence of prothrombin, and none was detected (data not shown).

Analysis on nondenaturing gels. Lentil lectin eluant was electrophoresed in a continuous acrylamide gel system and parallel samples were either silver stained or fractionated and assayed for carboxylase activity. Only a single silver-staining band was observed, and this band comigrated with the only peak of carboxylase activity observed (Fig. 3). To make a correlation between the single silver-staining band observed on the native gel (Fig. 3) with what had previously been observed in a denaturing discontinuous gel (Fig. 1), radioiodinated carboxylase was also electrophoresed in the nondenaturing gel system (data not shown). Protein isolated from the peak of radioactivity (which comigrated with the peak of carboxylase activity of a nonradioactive carboxylase sample run in a parallel lane) migrated as a 98-kDa band when analyzed on a denaturing gel. These results demonstrate that the 98-kDa band comigrated with carboxylase activity in the

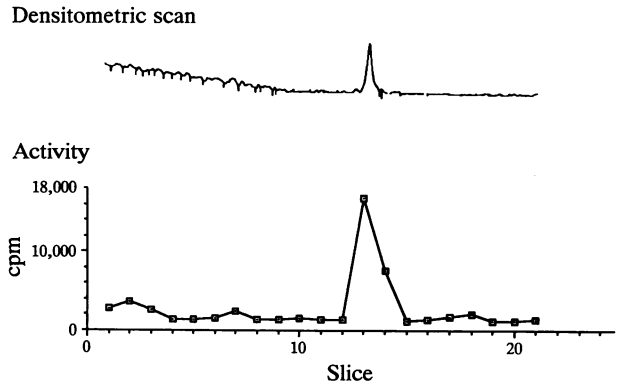

FIG. 3. Analysis of purified carboxylase on a nondenaturing gel. Multiple aliquots of lentil lectin eluant were electrophoresed on a continuous nondenaturing acrylamide gel. One part of the gel was silver stained and then scanned with an LKB laser densitometer. The other portion of the gel was fractionated and assayed for activity.

nondenaturing gel and strongly suggest that the 98-kDa band is the carboxylase.

DISCUSSION

We have purified the vitamin K γ-carboxylase 3.5×10^5-fold to obtain a single 98-kDa band that has carboxylase activity. The purification used here extends previous methods involving detergent extractions and the affinity of the carboxylase for vitamin K-dependent proteins and for the propeptide. Previous attempts to purify the carboxylase by conventional approaches yielded 100- to 400-fold purifications (11–13). The extent of purification of these earlier preparations was variable, however, and comparisons are difficult because of the use of different assay systems. For example, in the first purification step described here—i.e., the microsomal resolubilization—the choice of activity assay was critical in evaluating the extent of purification. With a previously described assay (14), the observed increase in specific activity between crude and resolubilized microsomes was 200-fold. However, gel analysis of the two fractions was consistent with the 2- to 3-fold increase determined by using the assay described here. The detergent extraction could have removed an inhibitor to which the assay used here is not sensitive, rather than significantly purifying the carboxylase.

To purify the carboxylase further, we followed detergent extraction and affinity purification with ion-exchange chromatography and fractionation using immobilized lentil lectin. S-Sepharose chromatography produced a reasonable increase (12-fold) in specific activity and was particularly useful for concentrating the very large volumes generated during propeptide elution. The carboxylase was shown to be a glycoprotein, and its fractionation on lentil lectin-Sepharose gave a substantial increase (22-fold) in specific activity. When the lentil lectin eluant was gel electrophoresed, a single 98-kDa band was observed.

Purifications of the carboxylase have also been reported by using the propeptide directly as an affinity reagent. Hubbard *et al.* (15), using propeptide affinity chromatography, reported the isolation of a 78-kDa protein with a specific activity of 10^4 cpm·hr^{-1}·μg^{-1}. In our preparations, a 78-kDa band, BiP, appeared as a major contaminant in the propeptide eluant. The presence of BiP in the eluant probably does not have any functional significance, at least *in vitro*, because removal of BiP from α-PT Sepharose with ATP had no effect on carboxylase activity.

Purification of the carboxylase using a propeptide plus Gla domain ligand has been described by Wu *et al.* (16). Their preparation yielded a prominent 95-kDa band, similar in size to ours. However, their -fold purification (7×10^3) is much

lower than that reported here (3.5×10^5). Activity determinations were done by comparable assays, excluding the assay as an explanation for the observed discrepancy. We and Wu *et al.* (16) have both based our protein determination on gel electrophoresed samples. In our studies, the same protein concentrations were obtained on crude microsomes using either scanned electrophoresed samples or BCA analysis. Moreover, the protein determination (by gel scanning) on purified carboxylase was similar to that obtained by amino acid analysis of trypsin-digested carboxylase (data not shown). Thus, the two independent methods used in this work for determining the starting and final protein concentrations are in agreement and argue that the carboxylase is a rare enzyme. Whether or not the proteins purified by Wu *et al.* (16) and us are identical will clearly be resolved by obtaining amino acid and/or DNA sequences.

To ensure that the 98-kDa protein we had purified was in fact the carboxylase, we used two different criteria. Antibodies prepared against partially purified carboxylase were used in a Western analysis of purified carboxylase, and the antiserum was immunoreactive with the 98-kDa band (Fig. 2). In addition, we demonstrated that the 98-kDa protein had carboxylase activity. Purified carboxylase was electrophoresed in a nondenaturing continuous gel system and a single silver-staining band was observed, which comigrated with carboxylase activity (Fig. 3). When radioiodinated carboxylase was electrophoresed on the nondenaturing gel, isolated, and then reelectrophoresed on a denaturing gel, the molecular mass of the single silver-staining band was determined to be 98 kDa. Because these two assays do not repeat methods used to purify the protein, they provide independent verification that the 98-kDa protein is the carboxylase. We have isolated a cDNA encoding this protein. The availability of the purified carboxylase, its cDNA, and carboxylase-specific antibodies will clearly facilitate the analysis of this unusual enzyme.

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